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Intestinal Absorption of Macromolecules during Viral Enteritis: An Experimental Study on Rotavirus-Infected Conventional and Germ-Free Mice

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ABSTRACT. Epithelial transport and degradation of horseradish peroxidase (HRP), a macromolecular tracer, was studied in conventional and germ-free suckling mice following an experimental infection with rotavirus. Conventional and germ-free mice developed diarrhea from days 2 to 8 postinfection (pi), with growth failure. In mucosal homogenates, infectious virus detected by immunofluorescence on MA 104 cells was present from day 2 through day 8 pi in germ-free mice, but persisted longer (day 13 pi) in conventional mice. Only mild histological lesions were observed during diarrhea, but obvious macrovacuolation of epithelial cells and increased cellular density occurred during the convalescence period (days 9 to 13 pi). Intact and degraded HRP fluxes from mucosa to serosa were measured *in vitro* on segments of jejunum mounted in Ussing chambers. Both groups of mice developed increased HRP permeability during the experimental period, but at different times after inoculation: during the diarrheal period (days 2 and 3 pi) conventional mouse epithelium absorbed five times more HRP than noninfected controls and during the convalescence period (days 9 to 13 pi) HRP absorption in germ-free mice rose 10-fold as compared to its level before infection. In both cases, this increase in HRP permeability was entirely due to an increase in intact HRP absorption, probably via a transcellular route, and occurred without any alteration in degraded HRP transport. These results indicate that in mice, rotavirus infection causes a transient rise in gut permeability to undegraded proteins. The intestinal microflora seems to affect the timing, magnitude, and duration of this increased permeability. (*Pediatr Res* 22: 72–78, 1987)

Abbreviations

HRP, horseradish peroxidase
pi, postinfection
EDIM, epizootic diarrhea of infant mice
PBS, phosphate-buffered saline
MEM, minimal essential medium
PD, potential difference
 I_{sc} , short circuit current
 ^3H -HRP, tritiated peroxidase

One of the major problems in the management of infant viral enteritis is to find a suitable diet during and after diarrhea. Of particular interest is the question of whether or not intact foreign proteins are overabsorbed during viral enteritis. Epizootic diarrhea of infant mice provides an interesting model for studying the mechanisms by which the epithelial barrier to macromolecules might be altered in viral enteritis. Intestinal permeability to macromolecular marker HRP was previously studied in isolated jejunal epithelium in different physiological and pathological situations (1–3) including coronavirus infection in piglets (4). Our earlier results indicate that HRP is transported through the enterocytes via two functional pathways: a minor pathway allowing intact protein transport and a major pathway including lysosomal degradation. In mice, these two pathways do not develop in a parallel manner during the neonatal period (5). Herein we report the gradual evolution of these two pathways during an experimental rotavirus diarrhea in suckling mice. This is one of the most frequently studied animal models, as it displays similarities to infantile gastroenteritis (6–8). Our results indicate that rotavirus infection in mice causes an increase in intact HRP intestinal permeability which is dependent on intestinal microflora.

METHODS

Animals. Germ-free and conventional C3H/HeJ mice supplied by Centre de Sélection et d'Élevage des Animaux de Laboratoire, Orléans-la Source, France, were used for experimentation. Germ-free mice were maintained in Trexler type plastic film isolators fitted with a rapid transfer system (La Calhène, Bezons, France). Dams and litters were transferred in an experimental isolator just before the infection of the pups with rotavirus. Infant mice were allowed to suckle throughout the period of experimentation. Birth day was taken as day 0. To avoid rotavirus cross contamination, conventional mice were housed in a building separated from the laboratory and from the experimentally infected animals. Conventional pregnant mice were screened for antibody to EDIM virus. Only litters from seronegative dams were studied. Both germ-free and conventional mice received γ -irradiated commercial diet (UAR, Villemoisson, France) *ad libitum*.

Rotavirus. A stock of infectious EDIM virus was prepared from intestines of infected C3H infant mice. Intestines were homogenized for 1 min at 4° C, in PBS pH 7.2 and Freon 113 (1.5 to 1.0 vol) using an Ultraturax homogenizer. After centrifugation (10 min, 2400 \times g), the supernatant was preserved. The interface was extracted again in 1 vol PIPES buffer pH 6.3 and centrifuged at 2400 \times g. Both supernatants were pooled, sterilized on a 0.22 μ millipore filter, and constituted the virus stock solution. This stock solution titrated 10^3 as determined by immunofluorescent assay on MA 104 cells.

In young mammals, including humans, rotaviruses cause enteritis with a high rate of morbidity and sometimes mortality.

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Infection of infant mice and specimen collection. Litter size was five to seven infant mice. All pups were given 20 μ l of the EDIM virus stock solution orally on day 5 after birth. They were allowed to suckle and checked daily for diarrhea by gentle pressure on their abdomens. At 2-day intervals after infection, one infant from one litter was killed by cervical dislocation. After laparotomy, the small intestine was excised and stretched out to its full length. It was divided into proximal and distal portions. Part of the proximal intestine was immediately processed for measurement of transmural HRP fluxes and the remaining parts were frozen until virus titration. Sometimes, one piece of intestine was fixed for histological examination (see below).

Histological examination. After fixing in Bouin's solution, segments of the proximal small intestine were dehydrated, embedded in paraffin wax, sectioned and stained with alcian blue and periodic acid-Schiff reagent.

Virus assay. The fetal rhesus monkey kidney cell line MA 104 was grown in Eagle's MEM supplemented with 8% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/ml, streptomycin 100 μ g/ml). Two days before assay, the cells were seeded at $3 \cdot 10^4$ cells per well in microtitration plates (Falcon, cat. 3072). Immediately before assay, the plates were washed twice with MEM without serum. Frozen intestinal segments of infected infant mice were thawed, adjusted to 0.6 g/ml (wet weight) in 25 mM Pipes buffer pH 6.3, homogenized, and centrifuged (1 min at $17,000 \times g$). Serial 10-fold dilutions of the supernatant (10^{-2} to 10^{-7}) in MEM supplemented with 3 μ g/ml trypsin (Difco, OSI, cat. 5015213) and antibiotics were transferred to triplicate wells. Positive and negative reference samples were included in each test. After 48 h of incubation, the plates were fixed with chilled ethanol-acetone (3/1) and kept at -20°C until tested in immunofluorescence. The plates were air dried, rinsed with PBS containing 0.05% Tween 20 (PBS Tween), and a rabbit anti-EDIM hyperimmune serum (diluted 1/500 in PBS Tween), raised against purified EDIM virus, was added for 1 h 30 min at 37°C . They were then washed with PBS Tween and incubated (1 h 30 min, 37°C) with a fluorescein-conjugated goat anti-rabbit IgG (diluted 1/200 in PBS Tween, Institut Pasteur production). After final washings with PBS Tween, fluorescence was scored from 4+ (many positive cells per well) to 1+ (5 to 10 positive cells per well) and the titer expressed as the reciprocal of the last dilution still yielding a positive score (mean of triplicates).

Enzyme immunoassay for serum Ig antibody to rotavirus. Dams were bled from the retro orbital venous plexus before infection of their litters with rotavirus. The presence of IgG antibody to rotavirus was determined by an Elisa immunoassay (9) on microtiter plates coated overnight at $+4^\circ\text{C}$ with a purified tissue culture adapted rotavirus (100 μ l of a 5 μ g/ml solution per well) in 0.01 M bicarbonate buffer pH 9.6, washed with PBS containing 0.05% Tween 20, and exposed to 150 μ l serial dilutions of the test sera. After 2 h incubation at 37°C , and extensive washings in PBS-Tween, an anti-mouse IgG-HRP conjugate (Institut Pasteur production) was added to each well (100 μ l of 1:750 dilution in PBS Tween) for 2 h at 37°C . After final washings, plates were exposed to *O*-phenylenediamine in 0.3% hydrogen peroxide for 15 min. The reaction was stopped with 5 N H_2SO_4 and the color produced was measured at 490 nm. Wells coated with antigen (Bovine rotavirus) and exposed to the appropriate enzyme-linked antiimmunoglobulin in the absence of mouse serum served as background controls. Titers lower than 1:27 were considered as negative.

Transepithelial HRP fluxes. At 2 days intervals during the 2 wk following infection, a pup was sacrificed in a litter and a 0.5-cm segment removed from the jejunum was used for HRP transport study. The segment was opened as a flat sheet, gently rinsed with cold Ringer and laid on a Millipore filter (HAKK 0.45 μ m). Filter and tissue were mounted in a small Ussing chamber with an oval-shaped opening between the mucosal and the serosal compartments, as previously described for human jejunal biopsies (2). Tissues were not stripped of their muscular layer. The exposed surface area was 0.1 cm^2 . Silicone paste

(Rhodorsil, Rhone-Poulenc, France) was used to minimize edge damage. Both sides of the tissue were bathed with 1.5 ml of Ringer solution, pH 7.4, thermostated at 37°C and oxygenated (95% O_2 , 5% CO_2) containing (in mM): 140 Na^+ , 5.2 K^+ , 1.2 Ca^{2+} , 1.2 Mg^{2+} , 120 Cl^- , 25 HCO_3^- , 2.4 HPO_4 , 0.4 H_2PO_4 , and 5 glucose. The solutions bathing the mucosal and serosal sides of the tissue were connected via 3M KCl agar bridges to calomel electrodes for measurement of the transepithelial PD and to Ag-AgCl electrodes for the passage of current through the system. Open circuit conditions were used in HRP flux experiments. Tissue conductance (G) was assessed regularly by passing a current through the preparation. Isc was then calculated, taking into account the fluid and filter resistance. HRP (Sigma, Type VI) at a final concentration of 10 μM (0.4 mg/ml) was added to the mucosal compartment. ^3H -HRP, prepared and stored as previously reported (1), was also added as a tracer (1.35 μCi). For 110 min, 800- μ l samples were taken from the serosal compartment at 10- and 20-min intervals and replaced by fresh buffer. The rate at which intact HRP was transferred to the serosal side was determined by enzymatic assay of 200- μ l aliquots according to a modified version of the Worthington method (10). Results were expressed as $\text{pmole} \cdot \text{cm}^{-2}$ intact HRP accumulated in the serosal compartment after 110 min of experimentation. Intact plus degraded HRP transport was assessed on 500- μ l aliquots by tritium counting using liquid scintillation photometry and expressed as the ^3H -HRP equivalent accumulated at 110 min (1). Degraded HRP transport was calculated as the accumulation of ^3H -HRP equivalent minus intact HRP after 110 min of experimentation.

Statistical analysis. Differences between infected and control mice were assessed by Student's *t* test for comparison of means and ranges.

RESULTS

Clinical. All 69 infected pups had diarrhea but none died during the experimentation; no differences could be detected between conventional and germ-free mice on diarrhea kinetics, as previously reported (11). Diarrhea consistently began on day 2 pi, declined on day 8 and disappeared by day 9. The quantity

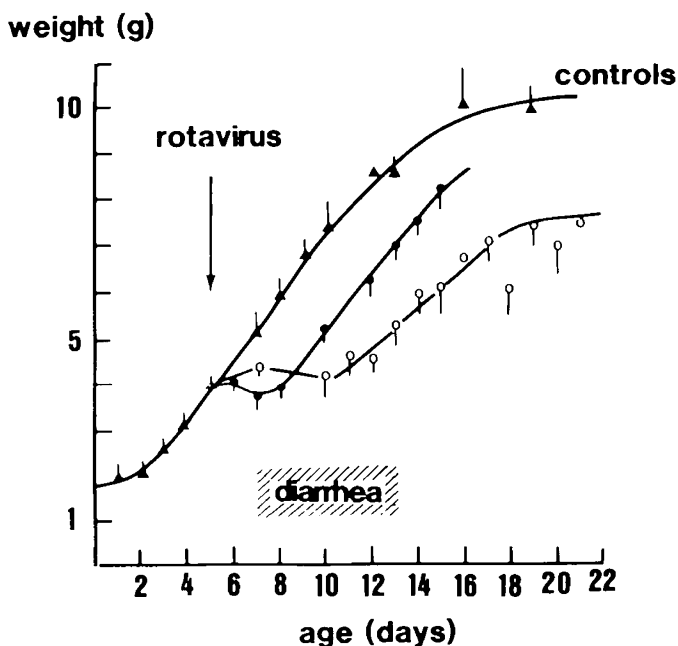


Fig. 1. Growth rate curve. Growth rate of control (▲) and infected conventional (●) and germ-free (○) suckling mice. Values were pooled as the growth rate was similar in conventional and germ-free control pups. Rotavirus infection was performed on day 5.

of feces varied among litters and sometimes within the same litter. Control and infected animals were weighed at various intervals and growth rates are presented in Figure 1. Uninfected conventional and germ-free suckling mice showed the same growth rate. Growth rate deficiency in rotavirus-infected animals was more marked in germ-free ($n = 35$) than in conventional mice ($n = 29$) except in the initial phase. Between days 5 and 13 pi, differences were significant between controls and infected conventional mice ($p < 0.05$) and between infected conventional and infected germ-free mice ($p < 0.05$).

Infectious virus in intestinal homogenates. Germ-Free Mice. Infectious virus reached a high titer (10^4 , 10^5) as early as day 2 pi in the proximal and distal small intestine. This high titer

persisted until day 7 pi and fell abruptly as no more virus was detectable in homogenates collected on day 8 pi (Fig. 2A). Infectious virus titers seemed to decline earlier from the proximal intestine between days 5 and 7 pi, probably indicating the proximo-distal development of the infection as suggested by others (12).

Conventional Mice. The pattern of infectious virus excretion was not similar to that observed in germ-free mice (Fig. 2B). Intestinal homogenate titers were not significantly different from those of germ-free mice during the diarrheal period ($\log_{10} = 4.55 \pm 0.43$ and 4.45 ± 0.41 , respectively), but significantly higher ($p < 0.001$) during the convalescence period ($\log_{10} = 3.39 \pm 0.37$ and 0.88 ± 0.36 , respectively). There was no correlation between

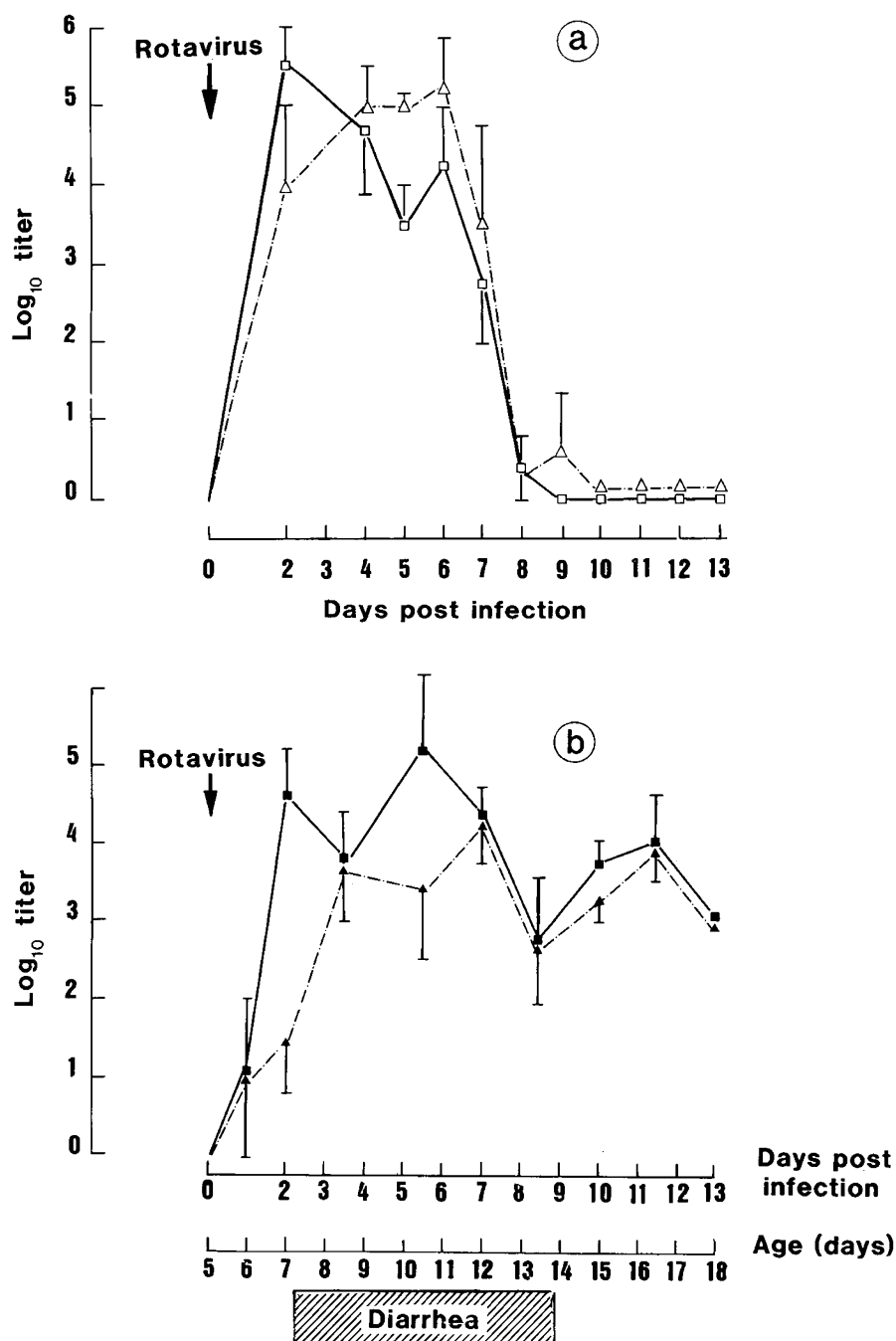


Fig. 2. Rotavirus titration. Infectious virus present in proximal (□, ■) and distal (Δ, ▲) mucosal homogenates of germ-free (A) and conventional (B) mice expressed as \log_{10} of the average highest dilution capable of infecting MA 104 cells as determined by immunofluorescence assay. Each point represents two to five suckling mice from different litters.

diarrhea and infectious virus in the small intestine since diarrhea disappeared by day 8 pi when infectious virus titers were still high.

Histological examination. In both conventional and germ-free mice, intestinal samples were only examined at the jejunal level. During diarrhea when infectious virus titers reached maximal values in germ-free mice, small histological lesions were revealed by light microscopy (Fig. 3*A*) compared to a control section of

an age-related mouse (Fig. 3*B*). Some microvacuolation of the enterocytes was noted, as well as a large release of mucus as indicated by the absence of dark stained goblet cells in infected compared to control tissues. Brush border membranes appeared to be regular and the integrity of the epithelial layer was preserved. The main histological modifications occurred from day 8 pi, with the appearance of large vacuoles in the enterocytes of the villi and an important increase in cellular density along the

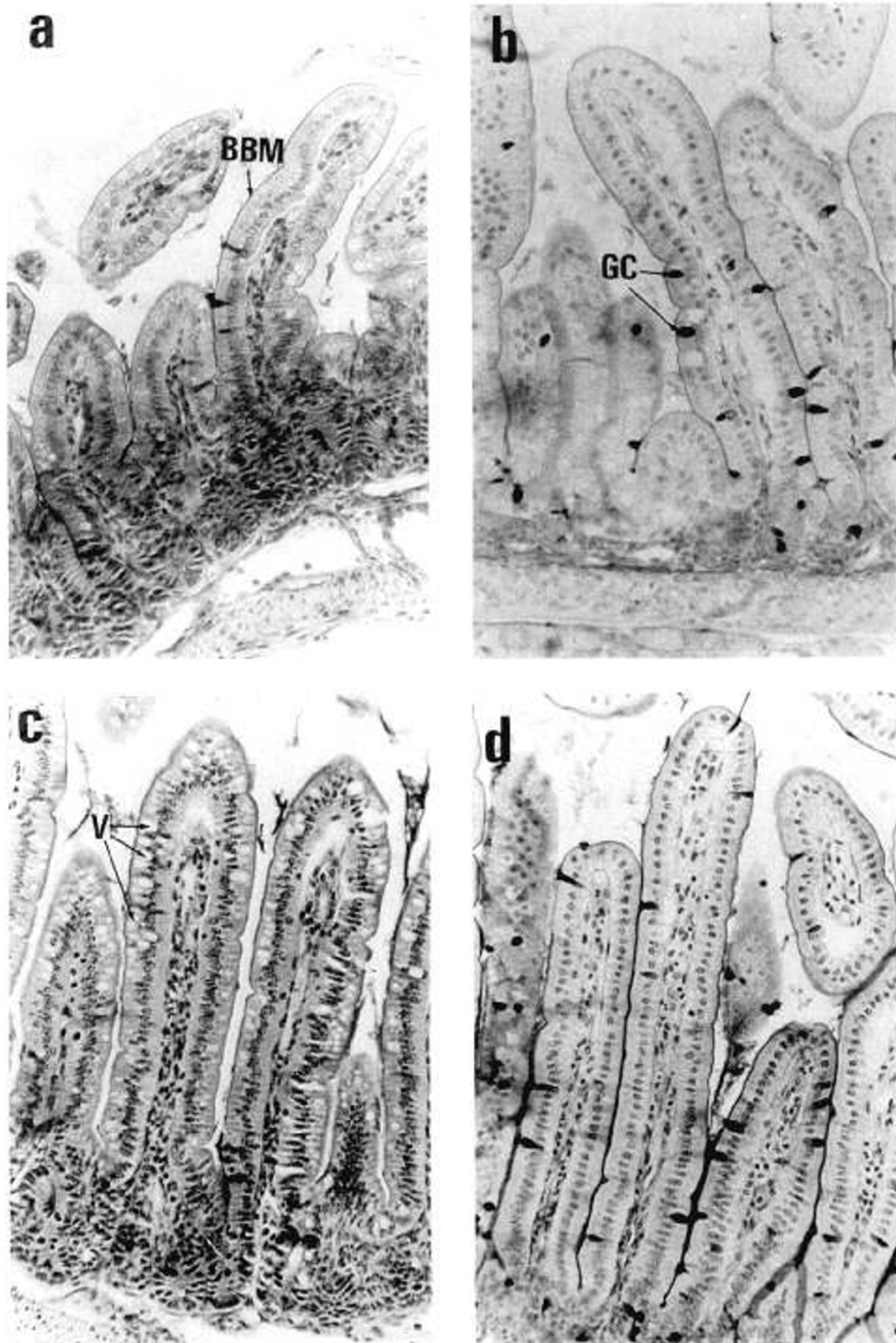


Fig. 3. Histological examination. Histological appearance ($\times 400$) of the jejunal mucosa of germ-free suckling mice examined on (A) day 5 pi (age 10 days) when virus was present in the mucosa compared to (B) an age-related noninfected mucosa and on (C) day 8 pi (age 13 days) when virus was no longer present in the mucosa compared to (D) an age-related noninfected mucosa. Note that brush border membranes (BBM) remain regular during the infection and that goblet cells have released their mucus content (A) and (C), whereas control epithelia (B) and (D) appear with dark stained goblet cells (GC). Note also that the epithelium lining the villus of convalescent mice (C) present large vacuoles (v) and higher epithelial cellular density than the related control epithelium (D).

crypt-villus axis, suggesting accelerated cellular proliferation (Fig. 3C). Jejunal epithelium from conventional mice presented the same characteristics although increased epithelial cellular density and macrovacuolation during convalescence were not as marked as in germ-free mice.

Using chamber experiments. Electrical Parameters of Jejunal Tissues. Neither conventional nor germ-free infected mice displayed significant modifications of electrical parameters during diarrhea or convalescence as compared to noninfected controls (Fig. 4). As previously reported (5), germ-free control animals exhibited lower *I*_{sc} and total ionic conductance (*G*) than conventional control mice. During viral enteritis, total ionic conductance (*G*), which is an index of paracellular permeability, did not increase in either group, indicating that the epithelial layer had not been damaged. In the conventional infected mice, ionic conductance even declined to values not significantly different from those of germ-free mice.

HRP Transepithelial Fluxes from Mucosa to Serosa. Germ-free mice. Figure 5 shows that throughout the diarrheal phase (days 2 to 8 pi), when the virus was present in the epithelium, there was no change in mucosal-to-serosal permeability to HRP, either intact or degraded. By contrast, during convalescence (days 9 to 14), when the virus was no longer present in the mucosa, we observed a 10-fold increase in intact HRP transport (Fig. 5A) but no modification of degraded HRP transport (Fig. 5B). Although individual variations between suckling mice were large, both within the same litter and between litters, this increase was

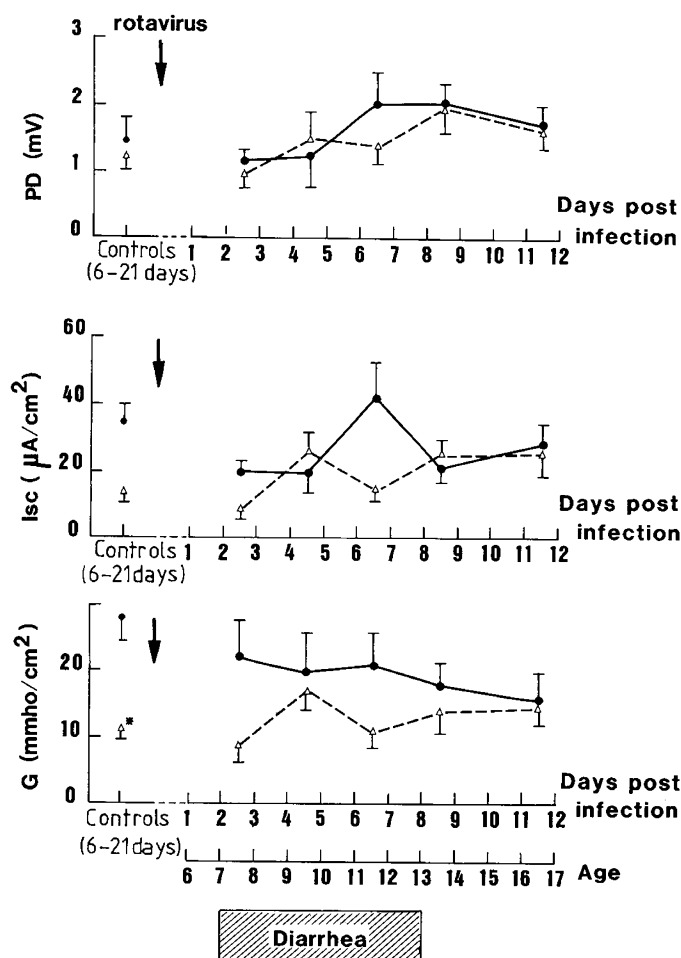


Fig. 4. Electrical parameters of intestinal tissues. Potential difference (PD), *I*_{sc}, and conductance (*G*) in jejunal epithelium of rotavirus-infected conventional (●) and germ-free (Δ) suckling mice as a function of the number of postinfection days. Control values are the means obtained for age-related suckling mice (6 to 21 days) as previously reported (5).

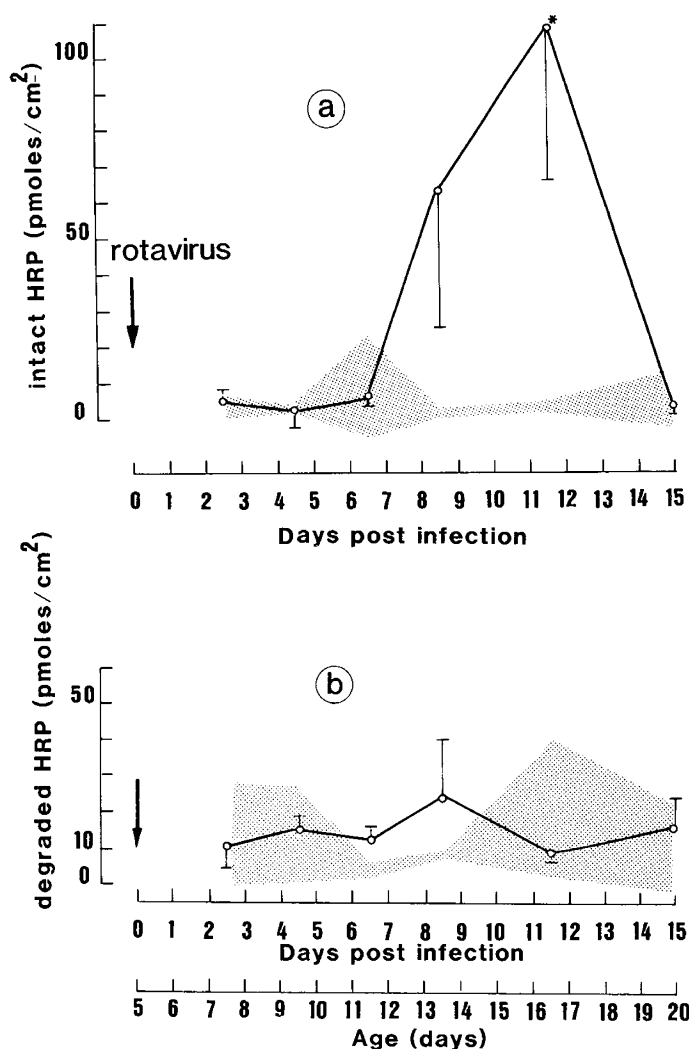


Fig. 5. A and B, HRP transport in germ-free mice. Time course of intact- (A) and degraded- (B) HRP accumulation in the serosal compartment of control and infected germ-free mice. Each point represents six infected mice and the hatched area represents the confidence limits for control mice (mean \pm 1.96 SEM).

statistically significant from day 10 pi ($p < 0.05$). Permeability returned to normal by day 14 pi.

Conventional mice. Figure 6 shows that the presence of intestinal microflora completely altered the pattern of HRP permeability during viral enteritis. Thus, intact HRP fluxes from mucosa to serosa rose 5-fold ($p < 0.05$) for a short period early after infection (days 2 and 3) but returned to normal values throughout the diarrheal or convalescence periods. As in germ-free mice, this increase was exclusively due to the rise in intact HRP transport (Fig. 6A), and there was no significant change in degraded HRP absorption (Fig. 6B).

DISCUSSION

Gastrointestinal infections associated with diarrhea have been suggested to alter mucosal permeability to intact macromolecules, possibly inducing subsequent sensitization to dietary proteins in predisposed subjects (13, 14). However, data on changes in intestinal permeability to intact proteins during acute enteritis are still very controversial, perhaps because gastroenteritis etiologies have not been accurately defined. In a previous investiga-

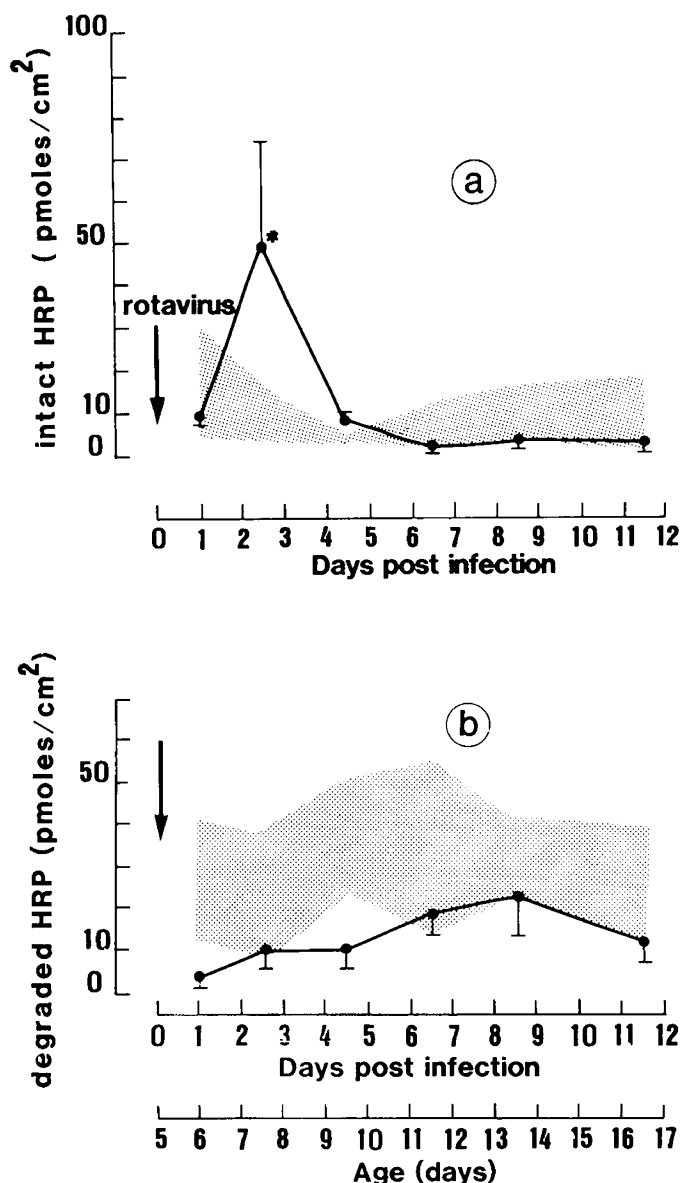


Fig. 6. *A* and *B*, HRP transport in conventional mice. Time course of intact (*A*) and degraded (*B*) HRP accumulation in the serosal compartment of control and infected conventional mice. Each point represents five infected mice and the hatched area represents the confidence limits for control mice (mean \pm 1.96 SEM).

tion, we demonstrated that experimental secretory diarrhea (cholera type) was not associated with increased intact protein absorption (3). In a recent study, Keljo *et al.* (4) reported that conventional piglets infected with transmissible gastroenteritis virus developed a marked increase in HRP permeability 12 h pi when structural lesions of the mucosa were observed. In the present study, we correlated the time course of another animal model of viral enteritis with changes occurring in intestinal protein uptake and degradation according to the microbial environment. In mice, the peak age for rotavirus infection is between 6 and 11 days (15). The severity of the disease depends on the nutritional status (16, 17), the local immune system, and the availability of virus specific receptors on the enterocytes (12, 15, 18, 19). The reason why we chose to work with conventional and germ-free mice was to examine the effect of a viral infection (to the exclusion of any other coincidental infection) and to establish the role, if any, of intestinal microflora on the time course of this infection as well as any related modifications of

intestinal protein transport. In a previous examination of the developmental pattern of protein absorption in conventional and germ-free suckling mice (5), we found a short transient increase in intact protein absorption at 3 and 4 days of age, but no further modifications until adulthood. The same pattern was observed both in conventional and germ-free mice, but the latter transported four times fewer proteins than their conventional counterparts. This new finding indicates that intestinal microflora has an important role, be it direct or indirect, in absorptive processes. As the intestinal permeability to HRP did not vary postnatally from day 5 to 21, we chose to infect our pups on day 5 in order to exclude any developmental variations in macromolecular absorption. In the present study, intestinal permeability to intact HRP greatly increased in the course of viral enteritis in infant mice. This increase was transient and occurred early after infection in conventional pups and later in germ-free pups. Furthermore, when intestinal microflora was absent, clinical and physiological disturbance were more severe, *i.e.* larger weight losses after rotavirus infection, and a more marked and long lasting augmentation in intestinal permeability to intact proteins. Theoretically, these differences in the clinical pattern of viral enteritis in the presence or absence of intestinal microflora might be due to the incidence of an immune protection of conventional mice, since seropositive mothers are known to protect their suckling pups which consequently exhibit less severe infections (12, 20). However, in the present study we used only seronegative mothers so that a protection of the pups by their mother did not seem to interfere. On the contrary, infectious virus was present longer in mucosal homogenates of conventional pups.

In infected conventional and germ-free mice, increase in HRP permeability was entirely due to an increase in intact HRP transport without any change in the lysosomal pathway. Although individual variations were large, the increase in intact HRP absorption did not seem to be an artifact because such variations may be caused by the patchy nature of the infection on the jejunal surface. Indeed, only the very small segment of mucosa mounted in the Ussing chamber was assumed to reflect the entire jejunal surface. The possibility of nonspecific leakage of proteins through the damaged tissue can be excluded since no major histological lesions were detected by light microscopy. A large release of mucus was observed during the diarrheal period. Such a release had previously been described with cholera toxin (21). In addition, constant recording of total ionic conductance, an index of paracellular permeability, showed that it remained unchanged throughout the infection. This strongly suggests that the increased HRP permeability was not due to paracellular leakage but rather to transcellular absorption. The possible dissociation of the two protein transport pathways (direct and degraded) with the predominant involvement of the direct non-degrading pathway in the present pathological situation can be explained in two possible ways. This could be due either to the appearance of a new cell type pool that transfers HRP intact or to the increase of the minor direct pathway in cells that already possess the degrading pathway. The immaturity of the epithelial layer after the viral infection might also be a factor influencing the increased absorption of the undegraded HRP.

The second main finding of this study concerns the effects of intestinal microflora on the time course of changes in protein permeability. Our results indicate that conventional microflora does not play a role of barrier against the development of infectious viruses. However, the timing of virus excretion was different in conventional and germ-free mice since titers were still high after day 8 pi in conventional mice, while they were very small in germ-free mice. This suggests that there is no strict correlation between virus excretion and diarrhea, since diarrhea was observed from days 2 to 8 pi in both conventional and germ-free mice. The differences observed in the alteration of macromolecular transport during the time course of the disease, in conventional and germ-free mice, underlines the importance of the microecological environment of the host on the response to

the infection. This might have clinical significance in bacterial disorders, *e.g.* during treatment with an antibiotic that destroys the predominant intestinal flora, since such a situation may enhance the alteration of the intestinal barrier to macromolecules.

All these results suggest that in suckling mice an intestinal viral infection may modify the epithelial transport and degradation of luminal antigens. The enhanced absorption observed during viral disease is more marked in the absence of intestinal microflora than in its presence. It remains to be determined whether such an alteration occurs in human infants with rotavirus infection and to establish whether increased intestinal absorption of intact proteins during viral enteritis can explain milk intolerance and delayed clinical recovery in children with diarrhea.

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